PCT/ SE 03 / 0 0 2 7 7

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(21) *Patentansökningsnummer* / *Patent application number* — *0200531-2*

(86) *Ingivningsdatum* / *Date of filing* — *2002-02-22*

Stockholm, 2003-02-26

För Patent- och registreringsverket
For the Patent- and Registration Office

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Novel biomolecular complexes, method for their production and use

The present invention concerns biomolecular complexes and in particular a method for linking biomolecules in sets of two or more, with predefined three-dimensional orientation and spacing between said two or more biomolecules. Said linking method has application in the construction of novel, highly specific vehicles for drug delivery, for example in gene therapy, and in the construction and performing of assays for the study of biomolecular interaction, such as protein-protein, and especially receptor-ligand, interaction studies.

Background of the invention

Drug target discovery today is to a large extent based on the design of individual effector molecules on the basis of known target structures. Modern drug design is also based on single receptor-ligand interactions. In nature this is often not the case, as is exemplified by many viruses, which interact with more than one receptor. HIV has been shown to bind to both CD4 and chemokine receptors such as CCR5, and adenoviruses have been shown to bind both to integrins and to the CAR-protein. In addition to these, additional receptors have recently been identified. It seems likely that this concept is general and that efficient uptake of particles, such as viruses, is caused by the combined interaction with two or possibly more receptors. It is known from the study of adenoviruses that one of the constraints in viral particle uptake is the positioning of the ligands for integrins and for CAR. Thus the shafts or antennae of the adenoviral particle need to be of a certain length in order for internalisation. For most two-receptor systems, very little is however known about the optimal distances between the ligands, their orientation and exact mechanisms of interaction.

Prior art

In the international application WO 00/15824, a transfer method for specific cellular localisation of nucleic acids is described. The method is based on the use of peptide nucleic acids (PNA) as binding elements on a carrier molecule, and functional elements coupled to said binding elements. According to WO 00/15824, the functional element /-s may be separated from the binding element /-s by a linker. The carrier molecule may also comprise one or more detectable marker elements, or labels, such as labels detectable by spectroscopic, photochemical, biochemical, immunological or chemical means.

WO 00/15824 also discloses a transport entity, e.g. for the transport of nucleic acids over biological membranes, said transport entity comprising two or more functional elements, preferably spaced by linkers. A linker, according to WO 00/15824, may be comprised of a polymer of a suitable number of amino acid residues, or any other suitable molecule which functions as a spacer element without interfering with the desired function of the functional elements. Using the transport entity according to WO 00/15824, is possible to mimic the different functions of viruses and microorganisms by attaching functions directly to a nucleic acid or any other biological molecule and/or complex to be transferred to a cell.

It remains an objective to make available a simple, fast and reliable method for the production of biochemical complexes.

One aim of the present invention is to make available a practical method for the production of biomolecular complexes with pre-determined and well-defined three-dimensional orientation and/or spacing between the biomolecules involved. Another aim is to identify receptor combinations that will provide improved or optimal uptake of drugs, and to construct vectors for drug delivery, including vectors for gene delivery.

Another aim is to make available a method and technology for receptor screening, and in particular for high throughput combinatorial receptor screening.

Another aim is to make available means and methods for the study of biomolecular interaction, such as protein-protein interaction studies, and especially receptor-ligand interaction studies.

It is also an aim of the invention to improve the binding and uptake of substances through biological membranes, both extra- and intracellular binding and uptake, using specific binding mechanisms.

Summary of the invention

The present invention makes available a biomolecular complex comprising at least two functional elements (FE_1, FE_2) each attached to a target molecule or target area (T) through binding elements (BE), characterized in that each FE is attached to a specific BE, said BE exhibiting selectivity for a specific target molecule / target area or a part thereof, and the target molecules / areas being separated from each other by a first linker or spacer (L) having

a pre-determined physical property. The invention also makes available methods for the production of such complexes, as well as methods involving their use.

The present invention as defined in the attached claims fulfils the above objectives and aims, and makes it possible to produce novel biomolecular complexes, by linking multiple receptor ligands in a well-defined way and screen for cell specific uptake of the targeted molecule.

Short description of the drawings

The invention will be disclosed in further detail in the following description, example and attached drawings, in which

Fig. 1 shows schematically two biomolecules or functional entities, FE_1 and FE_2, connected to target areas T_1 and T_2 through binding elements BE_1 and BE_2. The distance between the functional entities and/or their orientation is determined by the rigid linker L and the optional linkers l_1 and l_2.

Fig. 2 shows schematically an experimental set-up, used to investigate the hypothesised trans-phosphorylation of Btk (Burton's Tyrosine Kinase). This protein is attached to a binding element via a linker (l), having a second rigid linker (L) of defined length, joining two target areas T for connection to the binding elements. By varying the length of the rigid linker, as well as the orientation of the target areas, and the possible linkers between the target area and the protein, the spatial relationship between two protein molecules can be varied in order to elucidate the exact protein – protein interactions. This set-up is used to determine whether Btk can autophosphorylate or transphosphorylate itself.

Description

In the present description and claims, the following terms and abbreviations will be used:
In the following description, the term "functional element" (FE) will be used to denote any moiety capable of conferring one or more specific biological functions or properties to a molecule linked to it.

A "binding element"(BE) may be any natural or synthetic nucleic acid, nucleic acid derivative or nucleic acid analogue capable of specific, strong and durable binding to a specified target thereof, preferably by hybridisation. One example of such BE is the PNA described below.

A "target" or "target region" is a specific region corresponding to a BE, and may be any natural or synthetic nucleic acid, nucleic acid derivative or nucleic acid analogue capable of specific, strong and durable binding to a specified BE, preferably by hybridisation

A "linker" (L, l) may be any chemical structure connecting two BEs or an FE and a BE, defining a distance and orientation between these. Preferably a linker does not participate in the chemical / biochemical interactions of the FEs. The linker is preferably a nucleic acid polymer

"PNA" is an acronym for Peptide Nucleic Acid, which is a DNA mimic having a pseudopeptide backbone consisting of aminoethyl glycine units, to which the nucleobases are attached via methylen carbonyl linkers. A PNA molecule is capable of hybridising to complementary ssDNA, dsDNA, RNA and PNA targets. In the present application, it is to be understood that the term "PNA" refers to any DNA analogue comprising the above backbone and nucleobases, and the term is thus not limited to the specific structures disclosed herein.

A "label" or "marker" is a composition detectable by spectroscopic, photochemical, biochemical, immunological or chemical means.

The present invention makes a contribution over the prior art in that it makes available a novel complex and method for its production and use. The transport entity according to WO 00/15824 can be exemplified as

...BE-L-FE... (1)

wherein BE denotes a binding element, L denotes a linker (optional), and FE denotes a functional element.

The biomolecular complex according to the present invention, differs from the transport entity in that the position / orientation and/or distance between two or more functional elements is accurately controlled. The complex according to the invention can be illustrated as

FE_1-BE_1-T_1-L-T_2-BE_2-FE_2 (2)

wherein T_1 and T_2 denote target areas; L is a linker or spacer of predetermined length, or other specific quality; BE_1 and BE_2 denote binding elements; and FE_1 and FE_2 denote functional elements. The linker is optionally a rigid linker, depending on the intended use of

biomolecular complex. Each of the functional elements FE_1 and FE_2, the binding elements BE_1 and BE_2, and the corresponding target areas T_1 and T_2 form sub-units, each presenting one functional element. Optionally, FE_1 and BE_1, or FE_2 and BE_2 may be separated by secondary linkers (*l*), further determining the distance and orientation of the functional entities:

$$FE_1\text{-}l\text{-}BE_1\text{-}T_1\text{-}L\text{-}T_2\text{-}BE_2\text{-}l\text{-}FE_2 \qquad (3)$$

When the biomolecular complex comprises more than two functional elements, these are attached to binding elements corresponding to specific targets or parts of targets, optionally separated by further linkers / spacers. This complex can be illustrated by

$$[(FE_x\text{-}l\text{-}BE_x)\text{-}T_x]_n - L_m \qquad (4)$$

wherein n and m are integers, and where m may be equal to n, but frequently is equal to $n - 1$. The subscript x means that the functional entities and binding entities may be characterised by different functionalities or binding properties, respectively.

An embodiment of the present invention is a biomolecular complex comprising at least two functional elements (FE_1, FE_2 etc.) attached to target areas (T) through binding elements (BE), wherein each FE is attached to a specific BE, said BE exhibiting selectivity for a specific target molecule / target area or a part thereof, and the target molecules / target areas being separated from each other by a linker having a pre-determined physical property.

Another embodiment is a biomolecular multimer complex, where multiple copies of the same functional element, e.g. a ligand, are coupled to binding elements, and attached through these binding elements to multiple target molecules / target areas connected by linkers, e.g. a sequence comprising multiple target areas separated by sequence portions functioning as linkers.

According to the invention, the linker can be any suitable molecule, for example a polymer having specific physical properties and which does not interfere with action of the functional elements, other than their orientation and mutual distance. The physical property of the linker is one chosen among: length, charge, secondary structure, tertiary structure, hydrophilicity, or a combination thereof.

According to an embodiment, the target molecule / target area comprises a marker, such as a reporter gene, or a label, such as a fluorescent label.

According to one embodiment, the BE is a PNA sequence and the target molecule / target area comprises the corresponding PNA target.

One embodiment of the present invention is a method for the production of biomolecular complexes, comprising the steps of

a) forming a stock solution of a first functional entity,

b) forming a stock solution of a second functional entity,

c) forming separate stock solutions of at least two binding entities,

d) forming separate stock solutions of linker molecules, each solution containing a linker molecule having a distinct physical property,

e) reacting said first functional entity with at least one binding entity,

f) reacting said second functional entity with at least one binding entity, other than the binding entity in e)

g) repeating steps e) and f) for each functional entity,

h) reacting each linker molecule with at least two target molecules / target areas, capable of specific binding to the binding entities of e) and f)

i) reacting each combination of functional entity and binding entity with each linker, and

j) repeating step h) in order to form a library of combinations of functional entities and linkers.

Another embodiment of the present invention is a method for the production of biomolecular complexes, comprising the steps of

i) synthesis of a molecular combination of a first functional entity and a first binding entity,

ii) synthesis of a molecular combination of said first functional entity and a second binding entity,

iii) synthesis of a molecular combination of a second functional entity and said first binding entity,

iv) synthesis of a molecular combination of a second functional entity and said second binding entity,

optionally repeating steps i) – iv) for further functional entities and binding entities and forming stock solutions thereof,

v) synthesis of a linker connecting a first and second target area,

vi) self-assembly of the molecular combinations of any one of step i) – iv) to the linker of step v) in the desired configuration by addition of these to said linker in solution.

One further embodiment of the present invention is a method for the screening of receptors with respect to their involvement in the internalisation of substances in eukaryote cells, and in particular a combinatorial method, wherein complexes presenting different functional entities are produced according to the method outlined above, and subjected to analysis as outlined in the attached example.

One further embodiment of the present invention is a method for the screening of receptors with respect to their involvement in the internalisation of substances in prokaryote cells, and in particular a combinatorial method, according to the above.

Another embodiment is a method for the study of inter-molecular interactions under physiological or near-physiological conditions, wherein the molecule/-s of interest is/are inserted as one, two or more FEs, using a range of linkers varying the distance and orientation of the molecules in relation to each other.

According to one embodiment of the invention, single molecules such as ligands, can be broken down to separate peptides and the role and function of these peptides investigated by incorporating these peptides as functional entities in a biomolecular complex according to the invention. It can in this way be elucidated which part/parts of a ligand is involved in the interaction under investigation.

The present invention also encompasses specific drug delivery vectors produced using the inventive method, as well as drug candidates identified using the method according to the invention.

The inventive method makes possible the rational and easy manufacture of combinatorial libraries, consisting of large numbers of functional entities, such as receptors, or ligands for receptors, combined in pairs, three, or more, and connected by linkers having pre-determined physical properties.

The method according to the present invention has a significant advantage in that it makes possible the "self-assembly" of complex biomolecular structures.

The method according to the invention has the advantages of being based on relatively simple chemistry, making it possible to implement without large investments in production equipment and facilities.

The method according to the present invention has a considerable advantage in that no special buffers are needed, instead the reactions can be performed under physiological conditions. Further, no immobilisation is necessary when using the complexes, making the conditions of the reactions more similar to physiological conditions.

The method according to the present invention makes it possible to test different functional groups in different configurations, as the assembly of complexes having the desired spatial and functional parameters becomes easy.

Example

An experimental high through-put screening set-up

Initially, the peptides to be screened (PNA-X_{1-n}) matching target DNA, either plasmids or oligonucleotides, are synthesized carrying the PNA target sites. For this purpose, the Qiagen automation system (Qiagen Inc., USA) may be adapted for sequential hybridisation.

Each hybridization is then deposited in a well in a 96-well plate and heat-sealed. Again, equipment e.g. from Quiagen Inc. is available, having a robotic link system that can penetrate the heat-sealed well and then apply the sample to a predetermined well in a thin-bottomed 96-well plate with cell-cultures for further incubation.

Cellomics Inc. (Pittsburgh, PA, USA) manufactures a system for high-throughput screening of fluorescence in cells (Arrayscan II). The system can handle up to 20 96-well plates at each time. The system can control the culture conditions of the cells (temperature and CO_2) thus allowing for possible *in vivo* screening of the cells both of direct fluorescence detection of labeled nucleic acids and of GFP as a reporter for gene expression.

The Arrayscan II system is then used to incubate and screen the plates prepared as above.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention as set forth in the claims appended hereto.

Claims

1. Biomolecular complex comprising at least two functional elements (FE_1, FE_2) each attached to a target area (T) through binding elements (BE), **characterized** in that each FE is attached to a specific BE, said BE exhibiting selectivity for a specific target molecule / target area or a part thereof, and the target molecules / areas being separated from each other by a first linker or spacer (L) having a pre-determined physical property.

2. Biomolecular complex according to claim 1, **characterized** in that said at least two functional elements are the same, and that they are attached to a corresponding number of target molecules / target areas, separated by linkers, forming a multimer of said functional elements.

3. Biomolecular complex according to claim 1, **characterized** in that at least one of said functional element and corresponding binding element is separated from each other by a second linker (*l*).

4. Biomolecular complex according to claim 1, **characterized** in that the first linker or spacer is a nucleic acid polymer.

5. Biomolecular complex according to claim 1, **characterized** in that the second linker or spacer is a linker compatible with methods of peptide synthesis.

6. Biomolecular complex according to any one of claim 2 - 5, **characterized** in that the pre-determined physical property of the linker is chosen among: length, secondary structure, tertiary structure, charge, hydrophilicity /hydrophobicity, or a combination thereof.

7. Biomolecular complex according to claim 1, **characterized** in that the BE is a PNA sequence and the target area comprises the corresponding PNA target sequence.

8. Biomolecular complex according to claim 1, **characterized** in that the target area comprises a marker, such as a reporter gene, or a label, such as a fluorescent label.

9. Method for the production of biomolecular complexes, comprising the steps of

a) forming a stock solution of a first functional entity,

b) forming a stock solution of a second functional entity,

c) forming separate stock solutions of at least two binding entities,

d) forming separate stock solutions of linker molecules, each solution containing a linker molecule having a distinct physical property,

e) reacting said first functional entity with at least one binding entity,

f) reacting said second functional entity with at least one binding entity, other than the binding entity in e)

g) repeating steps e) and f) for each functional entity,

h) reacting each linker molecule with at least two target molecules / target areas, capable of specific binding to the binding entities of e) and f)

i) reacting each combination of functional entity and binding entity with each linker, and

j) repeating step h) in order to form a library of combinations of functional entities and linkers.

9. Method for the production of biomolecular complexes, comprising the steps of

i) synthesis of a molecular combination of a first functional entity and a first binding entity,

ii) synthesis of a molecular combination of said first functional entity and a second binding entity,

iii) synthesis of a molecular combination of a second functional entity and said first binding entity,

iv) synthesis of a molecular combination of a second functional entity and said second binding entity,

optionally repeating steps i) – iv) for further functional entities and binding entities and forming stock solutions thereof,

v) synthesis of a linker connecting a first and second target area, and

vi) self-assembly of the molecular combinations of any one of step i) – iv) to the linker of step v) in the desired configuration by addition of these to said linker in solution.

10. Method according to any one of claims 8 - 9, **characterized** in that the linker molecule comprises a marker or label.

11. Method according to any one of claims 8 - 9, **characterized** in that the binding entities are PNA sequences.

12. Method according to any one of claims 8 – 9, **characterized** in that the linker is a nucleic acid polymer.

13. A combinatorial library produced by the method according to any one of claims 8 - 9.

14. A combinatorial library according to claim 13, **characterized** in that the functional entities are chosen among receptor molecules, ligands for receptor molecules, or sub-units thereof.

15. Method for the screening of receptors with respect to their involvement in the internalisation of substances in eukaryote cells, **characterized** in that that a complex according to claim 1 is used.

16. Method for the screening of receptors with respect to their involvement in the internalisation of substances in prokaryote cells, **characterized** in that a complex according to claim 1 is used.

17. Method for the study of inter-molecular interactions under physiological or near-physiological conditions, **characterized** in that the molecules of interest is inserted as the functional entities (FE) in a complex according to claim 1, and the orientation and distance between the molecules varied by varying at least one of the first and second linker (L, l).

18. Drug delivery vectors produced using the method according to any one of claims 8 - 9.

19. Drug candidates identified using the method according to any one of claims 15 - 17.

20. Drug delivery vectors produced using a combinatorial library according to any one of claims 13 - 14.

21. Drug candidates identified using a combinatorial library according to any one of claims 13 - 14.

Abstract

The present invention concerns biomolecular complexes and in particular a method for linking biomolecules in sets of two or more, with predefined three-dimensional orientation and spacing between said two or more biomolecules. Said linking method has application in the construction of novel, highly specific vehicles for drug delivery, e g in gene therapy, and in the construction and performing of assays for the study of biomolecular interaction, e g receptor-ligand interaction studies.

(Fig. 1)

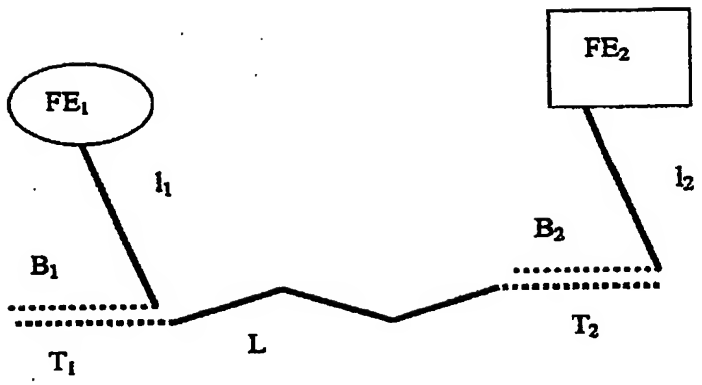

Fig. 1

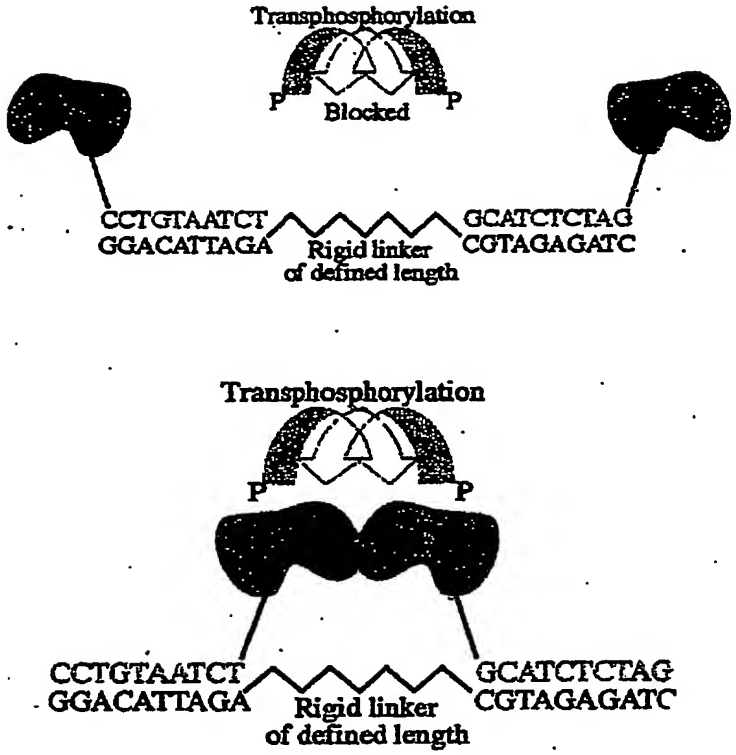
Transphosphorylation
P
Blocked
P
CCTGTAATCT
GGACATTAGA
Rigid linker
of defined length
GCATCTCTAG
CGTAGAGATC
Transphosphorylation
P
P
CCTGTAATCT
GGACATTAGA
Rigid linker
of defined length
GCATCTCTAG
CGTAGAGATC

Fig. 2